STUDY OF EXPRESSION OF p15PAF AND INTERACTION WITH miR-429 IN FIBROSARCOMA CELL LINE HT-1080

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BY
PRAGYAN PARAMITA SAHOO
ROLL NO.412LS2057

UNDER THE SUPERVISTION
OF
DR.BIBEKANAND MALLICK

DEPARTMENT OF LIFE SCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008, ODISHA, INDIA

राष्ट्रीय प्रौद्योगिकी संस्थान, राउरकेला
National Institute of Technology, Rourkela

Dr. Bibekanand Mallick, M.Tech., Ph.D.
Assistant Professor

RNAi & Functional Genomics Lab.
Department of Life Science
National Institute of Technology
Rourkela - 769 008, Odisha, India
Telephone: +91-661-246 2685 (O)
E-mails: vivek.iitian@gmail.com,
mallickb@nitrkl.ac.in
Website: http://vvekslab.in

CERTIFICATE

This is to certify that the thesis entitled "Study of expression of p15paf and its interaction with miR-429 in Fibrosarcoma Cell Line HT-1080" submitted by ***Ms. Pragyan Paramita Sahoo*** *(Roll No: 412LS2057) in partial fulfilment of the requirements for the award of* ***Master of Science in Life Science*** *to the National Institute of Technology, Rourkela, is an authentic and original record of research work carried out by her under my supervision and guidance.*

To the best of my knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

11/05/2014

(Dr. Bibekanand Mallick)

Dr. Bibekanand Mallick
Assistant Professor
Department of Life Science
NATIONAL INSTITUTE OF TECHNOLOGY
Rourkela-769008, Odisha

Place: *Rourkela*

Date: *11.05.2014*

DEDICATED TO

MY FAMILY AND BELOVED

ONES.....

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Date: PRAGYAN PARAMITA SAHOO

Place:

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ABBREVIATIONS

ACTB	β-Actin
µ	Micro
:	Ratio
%	Percentage
µl	Micro liter
cDNA	Complimentary DNA
PCR	Polymerase Chain Reaction
mRNA	Messenger RNA
DEPC	Diethyl Pyrocarbonate
Fig.	Figure
MFS	Myxofibrosarcoma
miRNA	microRNA
qRT-PCR	Quantitative real time PCR
et al.	And others

CONTENTS

ABSTRACT

MicroRNAs are a family of small, non-coding RNAs which regulate gene expression at post-transcriptional level. These ncRNAs influence cellular physiology by directly interacting with target gene transcripts. miRNA expression pattern can be correlated with cancer types, stages and other clinical variables. Therefore miRNA profiling can be used as a tool for cancer diagnosis and prognosis in almost all aspects of cancer biology such as proliferation, apoptosis, invasion and angiogenesis. Our study aims at identification of novel miRNA-mRNA target pairs that are hypothesized to play role in fibrosarcoma through an interaction map analysis from microarray data followed by experimental validation of selected pair of mRNA and miRNA by qRT-PCR. The target interaction map analysis revealed one novel target pair i.e. hsa-miR-429-p15PAF which can be potential therapeutic target in fibrosarcoma.

Keywords: miRNAs, fibrosarcoma, metastasis, non-coding RNAs

INTRODUCTION

Cancer is a complex disease that involves an array of changes at genetic & epigenetic levels and is characterized by uncontrolled cell growth. It affects millions of lives and also causes many deaths over the years. It consists of group of cells and is of more than 200 different types based on where they originate in the body. As cells are basic units of life so all organisms are composed of one or more cells. When the body requires cells, they start to divide to produce a number of cells in order to fulfill the requirement of the body. But when these cells keep dividing uncontrollably they create a mass of tissue. This mass of extra tissue is called as tumor which can be formed in all kinds of tissue and can be benign or malignant. Malignant tumors are called cancer. They can be of different types of based on their organ of origin like breast cancer, skin cancer, lung cancer, colon cancer, prostate cancer etc.

Sarcomas are the mesenchymal malignancies (D'Angelo et al., 2014) arising from bone, cartilage or connective tissues. Adult fibrosarcoma is defined by World Health Organization (Zambo and Vesely, 2014) as malignant neoplasm composed of fibroblasts having variable collagen production. It is also defined by Schultze et al as a tumor originating from mesenchymal cell which is composed of malignant fibroblasts with a collagen background (Schultze et al., 1998). It can occur as a soft-tissue mass or as a primary or secondary bone tumor. In the past though fibrosarcoma was diagnosed frequently but now it is more reliably distinguished histologically from similar lesions such as desmoid tumors, malignant fibrous histiocytoma, malignant schwannoma and high-grade osteosarcoma.

There are generally two forms of this disease:

- *Infantile or congenital fibrosarcoma*: A type of soft tissue sarcoma which is most commonly found in children less than one year of age (Ainsworth et al., 2014). At birth or shortly after birth, it presents as a rapidly growing mass. This form of fibrosarcoma is usually very slow-growing and also tends to be more benign than fibrosarcoma in older children, which behaves more like the type found in adults.

- *Adult form fibrosarcoma*: The adult form of this disease can occur in older children and in adolescents, roughly between the ages of 10 and 15. It is more aggressive than the infantile form and involves more complex treatment.

Though the exact cause of fibrosarcoma and other soft tissue tumors is not entirely known, however, studies indicate that genetic alterations may play a role. Limited studies have also shown a possible link between soft tissue sarcomas and the development of other types of cancer. A chromosomal rearrangement has also been found in some fibrosarcomas.

Though sarcoma may arise virtually anywhere, but the extremity is the most common primary site. During metastasis the most common site to which the fibrosarcoma spreads is the lungs. Of patients with extremity sarcoma, approximately 20% will have isolated pulmonary metastatic disease at some point in the course of their disease. Genetic mutation is the main cause of tumerogenesis and metastasis in soft tissue sarcoma. As mechanism for controlling metastasis are poorly understood so it is important to understand the molecular mechanism involved in primary tumor cell invasion and its spread to distant sites and thus to identify new molecular targets for cancer therapies.

Till date hundreds of miRNA have been identified in mammals some of which are expressed in a tissue specific and developmental stage specific way. In recent times much progress has been done in discovery of this regulatory RNA phenomenon. miRNAs generally interact with the target mRNAs (Than et al., 2013) with only partial or imperfect complementarity which causes either mRNA degradation or translational inhibition and thus can regulate the expression of target genes negatively. Thus up-regulation and down-regulation of miRNA has been seen to play a role in cancer progression.

In normal cellular development miRNA play essential roles, but functionally it may act either as oncogenes or tumor suppressors (Zhang et al., 2007) targeting analogous or tumor suppressor genes. miRNAs have the capacity to target gene transcripts directly and influence cellular physiology which is found to be involved in cancer etiology.

Though metastasis is well understood in cancers of epithelial origin, but it is not clearly understood in cancers of mesenchymal origin (sarcoma). So from microarray analysis we try to screen out probable genes responsible for metastasis in fibrosarcoma. In our present study, 1068

differentially expressed genes were obtained between Myxofibrosarcoma and the control normal fat samples. Of these 558 genes were up-regulated and 510 genes were down-regulated. Similarly 78 miRNAs were found to be differentially expressed in Myxofibrosarcoma and the control normal fat samples. Of these 27 were down-regulated and 51 were up-regulated. So from 558 up-regulated genes and 27 down-regulated miRNAs we wanted to check expression of p15PAF which is found to be targeted by miR-429 according to Magia[2] interaction map analysis.

REVIEW OF LITERATURE

Tumorigenesis is a multistep process wherein the normal cell is transformed into a malignant cell. The uncontrolled proliferation of these malignant cells by ignoring the normal rules of cell division results in disease known as cancer. Cancer is thus an abnormal condition which mainly results due to changes or mutation in the microenvironment of the cell. Cancer cell have the ability to spread into other body parts of the body and colonize and grow at the secondary sites. This process is known as metastasis. In 90% cases, metastasis is the main cause of death in patients suffering from cancer.

Cancer can be classified on the basis of where they originate or on the basis of location where they develop initially. Thus on the basis of tissues of origin cancers are of the following types:

Carcinoma: Cancer which arise from the covering cells of external and internal body surface called epithelial cells and these are the most common type of cancer.

Sarcoma: Cancer which arise from mesenchymal cells which are found in supporting tissues of the body for example bone, cartilage, fat, conective tissue and muscle.

Lymphoma: Cancers that arise in lymph node and tissues of body's immune system.

Leukomia: Cancers in which bone marrow is not allowed to form RBCs, WBCs and platelets and thus it is also called blood cancer (Appelbaum, 2011). WBC protects body from infection, RBCs help in oxygen transport and mainly prevent anemia and platelets help in clotting of blood at the time of injury.

Myeloma: This type of cancer is found to grow in plasma cells of bone marrow (Kyle and Rajkumar, 2009). It is of 2 types that is Plasmacytoma in which myloma cells found to accumulate in a sinle bone thus forming a tumor and second one is multiple myeloma where myeloma cells by accumulating in many bone produce many bone tumors.

Blastoma: It originates from immature precursor cells or embryonic tissue. It is found to be more common in children than in adult.

SARCOMA

These are the cancers of mesenchymal origin. In human sarcoma is quite rare. Majority of death in sarcoma is also due to metastasis. Here mainly 2 types of metastasis are there like nodal metastasis and distant metastasis. In nodal metastasis the cancer cell move to distant body parts through lymph node which is very rare one and other one is distant metastasis which generally spread to lungs.

Types of sarcoma:

1) Soft tissue sarcomas: These arise from soft tissues for e.g. muscles, fat, nerves, blood vessels and fibrous tissue. About 80% of sarcoma arises in soft tissues. According to WHO soft tissue sarcoma occupies 23rd position among all cancer on the basis of occurrence.

2) Osteosarcoma: It is otherwise called as osseous sarcoma which starts in bone. In comparison with benign bone tumor and secondary cancer, osteosarcomas are much less common. It occupies 27th position among all cancer on the basis of occurrence.

Again based on the cell type which makes up the cancer, sarcomas are subdivided into different types (Table 1).

Table 1. Subtypes of sarcoma and their origin:

Subtypes of sarcoma	**Type of cells forming tumor**
Angiosarcoma	Blood or lymphatic vessel
Chondrosarcoma	Cartilage cells
Ewing's sarcoma	Soft tissue or Bone
Fibrosarcoma	Fibrous tissue
Gastrointestinal stromal tumor tract	Gastrointestinal tract
Leiomyosarcoma	Smooth muscles of abdominal and pelvic organs

Liposarcoma	Fat tissue
Malignant peripheral nerve sheath tumor	Nerve and Spinal cord
Osteosarcoma	Bone
Pleomorphic sarcoma	Limb or abdomen
Rahbdomyosarcoma	Skeletal muscle
Synovial sarcoma	Often seen in young adults in different locations

Fibrosarcoma:

This is a tumor which develops from fibrous tissue which is mesenchymal in origin and is formed of fibroblasts, which are histologycally predominant cells (Loh et al. 2002). Fibrosarcomas form solid tumors. They may be of different grades according to the degrees of differentiation: low grade which is differentiated, intermediate grade and high grade (anaplastic). Depending on this differentiation, the tumour cells may resemble mature spindle-shaped fibroblasts which secrete collagen with rare mitoses. High grade mostly show metastasis that is they have the capacity to enter into local tissues thus spreading tumors to different body parts. Majority of death in fibrosarcoma is mainly due to metastasis which is poorly diagnosed or poorly understood in fibrosarcoma unlike in carcinomas.

METASTASIS

It is a process in which malignant cells move from primary tumor to distant organs through the lymphatic or haematogenous circulatory system. About 10% of deaths in patients of cancer occur due to primary tumor but 90% of death is due to movement of cancer cells (Leber and Efferth, 2009).

Metastatic sites

The sites where the primary tumor will spread and form a secondary malignant tumor is called site of metastasis (Table 2.). The determination of these metastatic sites by the tumor cells

in primary site is called organ selectivity. For organ selectivity 2 theories are there, first one is mechanistic theory in which organ is determined by pattern of blood flow and the other one is seed and soil theory (Fokas et al. 2007) that is spreading will occur to that site where there is fertile environment so that compatible tumor cells can grow (Nguyen et al. 2009).

Table 2. Different sites of metastasis

Primary tumor	Common distant sites
Breast adenocarcinoma	Brain, Bone, Adrenal
Prostate adenocarcinoma	Bone
Lung small cell carcinoma	Bone ,Liver, Brain
Skin cutaneous melanoma	Brain ,Liver
Testis carcinoma	Liver
Bladder carcinoma	Brain
Neuroblastoma	Liver ,Adrenal

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Steps of metastasis

Metastasis is a multi step process which involves the following steps (Fig 1)

1) Local invasion

2) Intravasation

3) Transport through circulation

4) Extravagation

5) Formation of micro-metastasis

6) Colonization

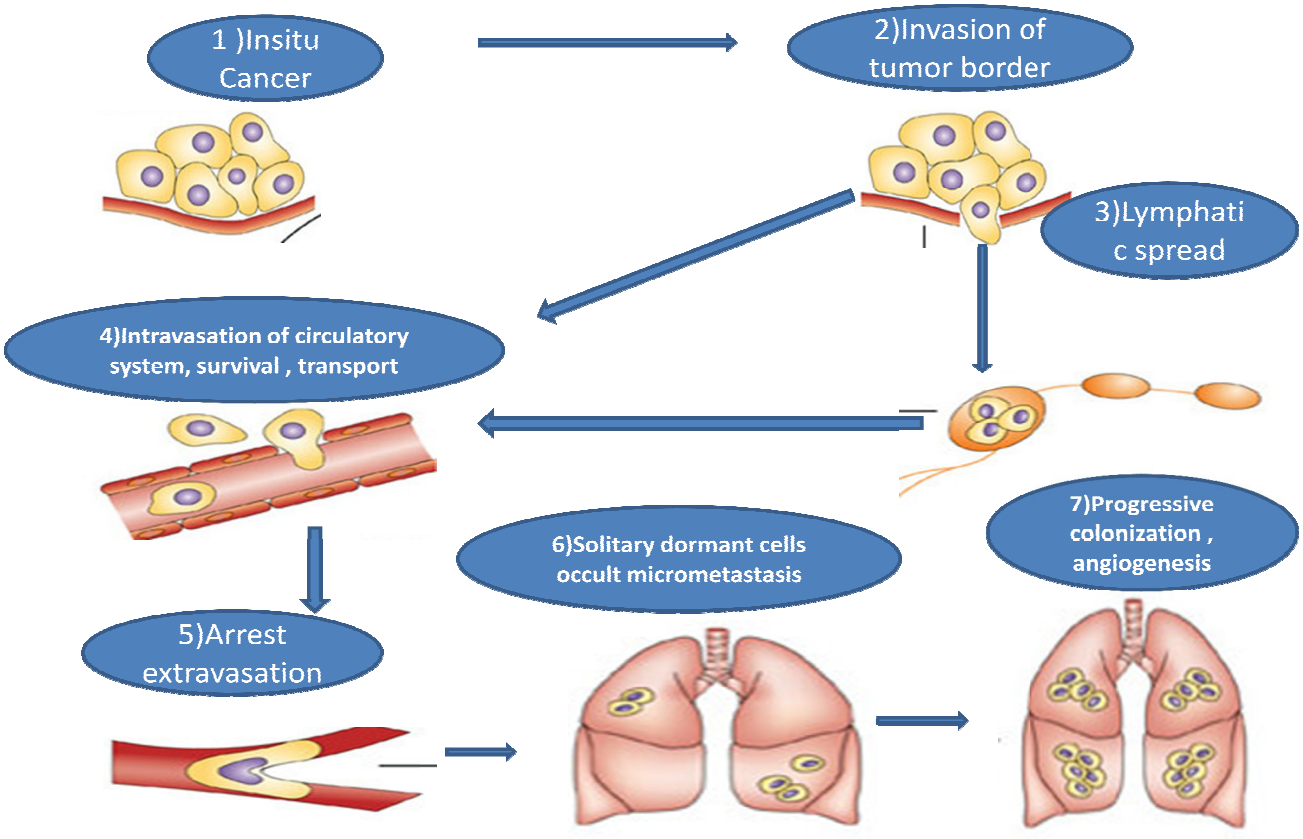

Fig 1: Different stages of metastasis

Local invasion: Through the basement membrane barrier, in-situ tumor breaks.

Intravasation: Passing through wall of capillaries the tumor cell move into circulatory system. In this step cancer cell will obtain different properties like invasiveness and cell motility. Due to this new property it can enter into the circulatory system.

Transport through circulation: Until and unless cancer cell will attach to a solid supporting tissue, it continues its journey through blood or lymph. Due to hostile condition at this stage many cancer cells will die or be destroyed and the remaining surviving cancer cell will attach to a first set of capillary and form microthrombi.

Extravasation: Finally in this step cancer cells will attach themselves to the secondary site and move into the tissue (Stoletov, 2010).

Formation of micrometastasis: After extravasation, the cancer cells have the capacity to reactivate cell proliferation pathways and finally a small tumor mass is formed at the secondary site.

Colonization: The new environment provides different factors for the survival and proliferation of cancer cell so as to colonize. This step is a challenging and complex one as the cancer cells have to adopt themselves to a completely new environment.

Molecules involved in metastasis:

For metastasis the tumor cell first have to acquire metastatic property which need a number of changes allowing intravasation and extravasation as well as colonization. A cell differentiated circuit is activated called EMT (Yilmaz and Christofori, 2009) that is epithelial to mesenchymal transition by promoting expression of a number of EMT permissive transcription factors. These cancer cells get the properties of mesenchymal cells that are invasiveness and motility. This process of EMT occurs mainly in cancers of epithelial origin. The different molecules involved are-

1) CAMs (cell-cell adhesion molecule) - It binds cells to surrounding tissue (Schlesinger and Bendas, 2014). They involve a protein called cadherin which help in metastasis and is seen in all epithelial cells. Generally in normal cells E-cadherin functions as a bridge between adjacent cells but in epithelial cancers function of E-cadherin is lost due to which it plays an important role in metastatic property (Onder et al., 2008).

2) Integrin- It is a protein having role in tissue invasion, angiogenesis, cell adhesion, migration, control of cell differentiation, proliferation and survival of cancer cells (Albelda, 1993).

3) MMPs (Matrix metallo proteinases) – Its increasing expression has role in successful colonization (Chambers and Matrisian, 1997).

Metastasis is one of the primary reasons for death in cancer patients. Recent reports suggest that microRNA also play an important role in regulating genes involved in different steps of metastasis.

MicroRNA:

They are small noncoding RNAs which control gene expression post transcriptionally through translational repression or degradation of mRNA (Cai et al. 2009). Recent reports suggest that microRNAs (miRNAs) play an important role in initiation and progression of different types of cancers. They are found to regulate different processes like cell differentiation, growth and apoptosis (Esquela and slack, 2006). For various diseases miRNAs are found to be biomarkers and potential therapeutic targets. In cancer cells deregulation of miRNA occurs by different mechanisms like amplification, deletion, mutation and epigenetic silencing (Garzon et al. 2009).

Maturation of miRNAs:

Primary miRNA (pri-miRNA) precursor molecule is produced by the transcription of miRNA (Fig 2). It undergoes cleavage producing precursor miRNA (pre-miRNA) which is cleaved to produce miRNA duplex in cytoplasm which contains mature miRNA. Then unwinding of duplex occurs as a result of which mature miRNA are released and assemble with RISC complex. These mature miRNA then direct gene silencing through translational repression or mRNA cleavage.

The intergenic miRNAs are transcribed with the help of either RNA polymerase II or III, (Fig 3). Pri-miRNA molecule is produced, which with the help of microprocessor complex made up of DGCR8 and Drosha give rise to pre-miRNA which with the help of nucleocytoplasmic transporter Exportin-5 and Ran-GTP is exported to the cytoplasm. Intronic miRNAs are transcribed with the help of RNA polymerase II forming pre-miRNA. From this pre-miRNA the miRNA sequence is removed in order to generate a Mirtron which is exported out of the nucleus.

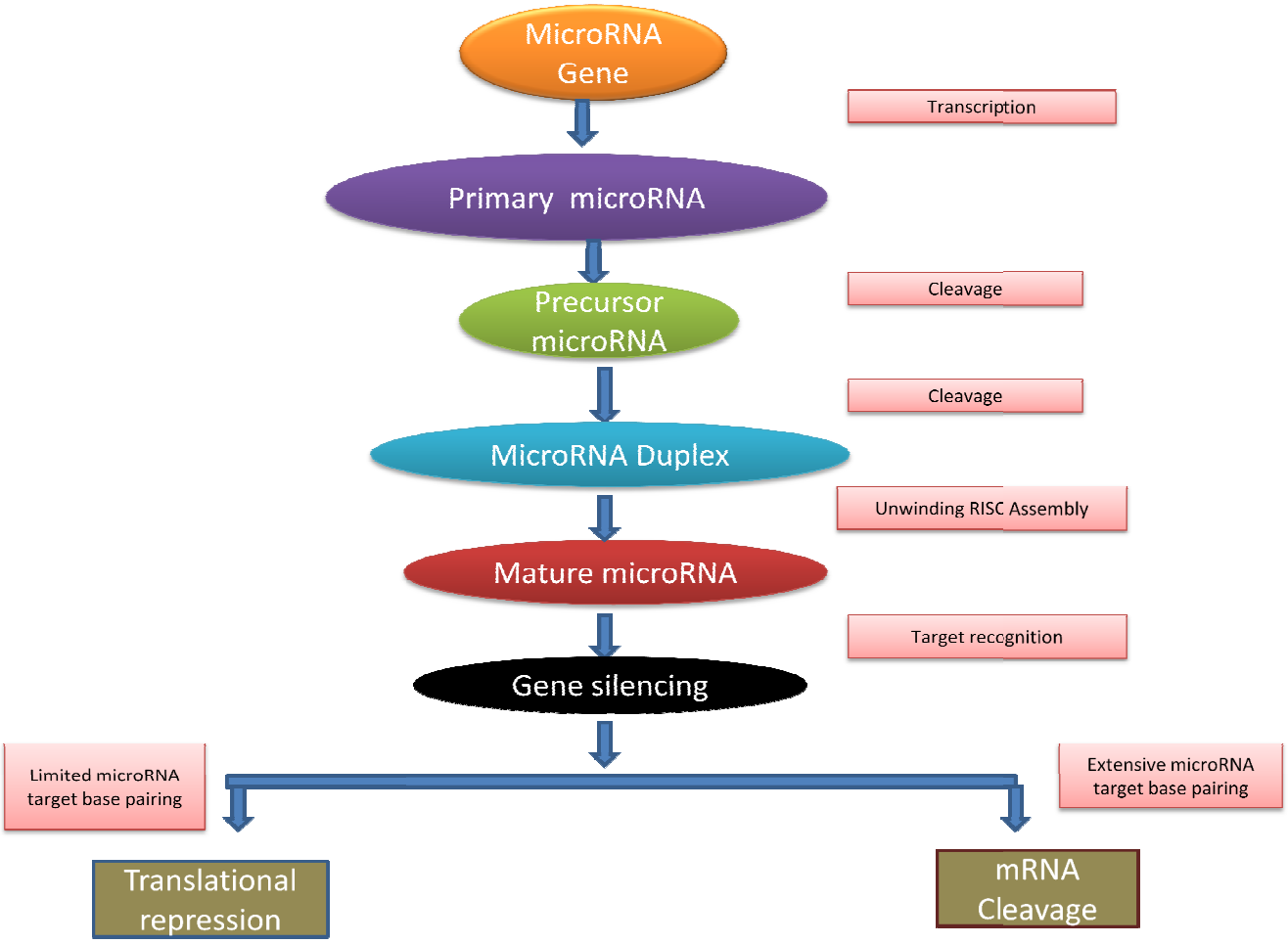

Fig 2: microRNA maturation and function

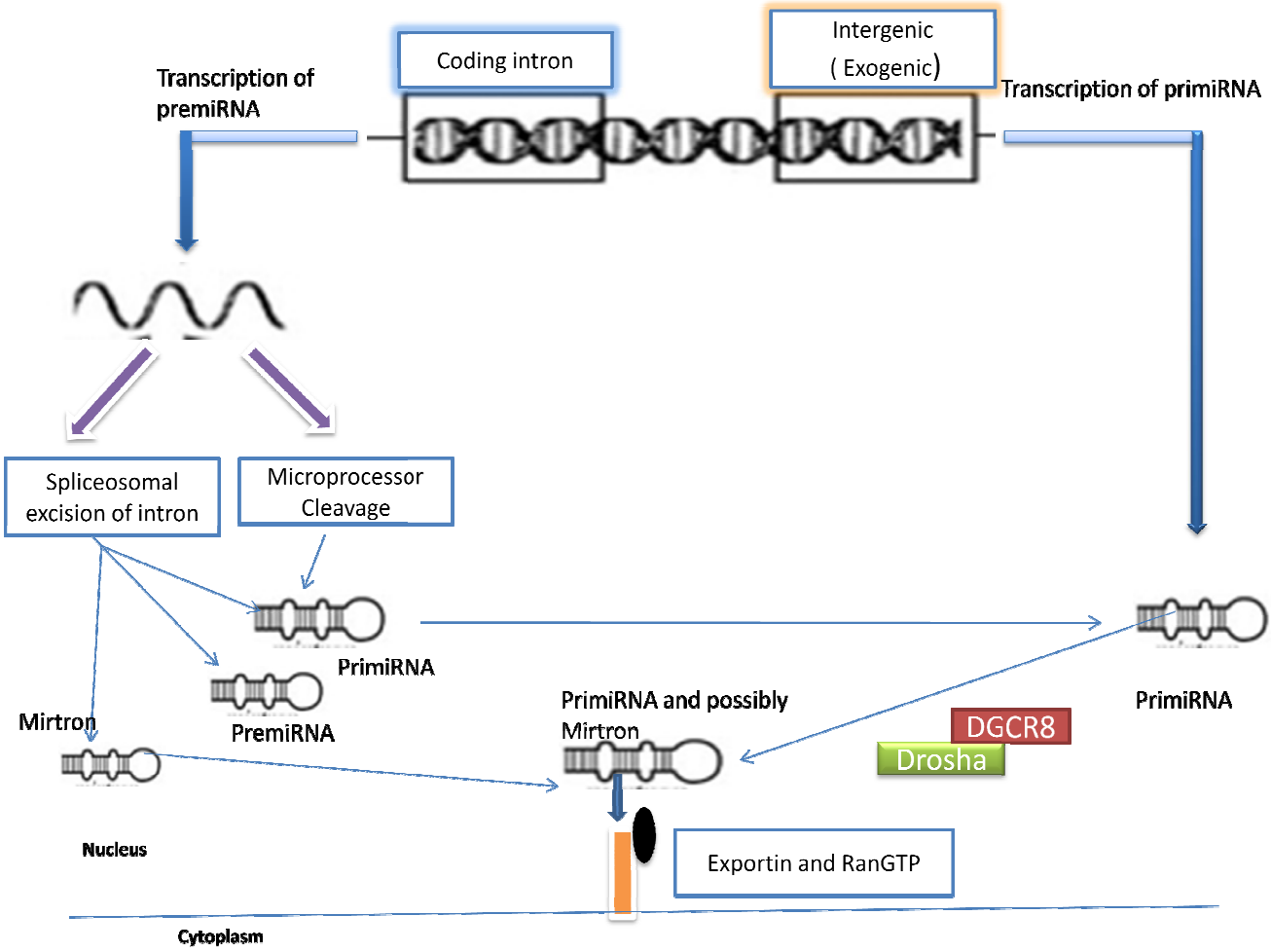

Fig 3: Different nuclear component taking part in microRNA biogenesis

MicroRNA targeting:

MicroRNAs generally target the mRNA for post transcriptional repression and for that it obeys certain rules. According to the rule, pairing of miRNA and mRNA needs a conserved Watson and Crick pairing towards 5' region of miRNA (Lewis et al., 2003). For post transcriptional repression miRNAs bind to 3' UTR of mRNA. Seed matching is not always helpful in predicting miRNA target which indicates that different other characteristics also help in specifying targeting. One third of human genes are found to be under selective pressure in order to maintain their pairing with that of the seed region of miRNA. Mainly 5' UTR of the

miRNA take part in binding but sometime in addition with 5’ UTR, 3’ UTR and open reading frame also take part in binding.

miRNA expression profiling in sarcomas

miRNA expression profiling in sarcomas has helped in the monitoring of the expression of thousands of miRNAs. Presently more than 1000 miRNAs have been identified in human. In different carcinomas, sarcoma and hematologic malignancies, analysis of miRNAs expression disclosed uniform pattern of specific miRNAs. Due to strong association with cancer some miRNAs are also called as oncomiR. For example miR-21 is found to be over expressed in gliobalstomas or microRNA 17-92 cluster found to be up-regulated in B cell lymphoma, rhadomyosarcoma and liposarcoma. Some miRNA clusters which are found to be associated with cancer or other disease are seen within chromosomal fragile regions resulting in sequence amplification or deletion. As sarcomas are highly heterogeneous in nature, it is very difficult to validate sarcoma diagnosis. So for proper diagnosis, prognosis and therapeutic intervention of different sarcomas miRNA signature plays an important role.

OBJECTIVES

Objective 1:

Microarray expression analysis in fibrosarcoma to identify set of differentially expressed mRNAs and miRNAs.

Objective 2:

miRNA and mRNA interaction map and target prediction for differentially expressed sets of miRNAs and mRNAs of fibrosarcoma.

Objective 3:

Identification of a novel miRNA–mRNA target pairs involved in fibrosarcoma and experimental validation by qRT-PCR in HT-1080 cell lines.

MATERIAL METHODS

1) Gene Expression Data:

For conducting genome wide analysis of mRNA and miRNA from normal and diseased sample in fibrosarcoma and in order to study and differentiate between different expression pattern for diagnosis and therapeutic mechanisms, the gene expression data is taken.

Gene Expression Omnibus is a data base from where the gene expression data were retrieved. The GEO is a common data storage supporting MIAME submissions that collects and distributes freely next generation sequencing, microarray and different forms of high throughput functional genomic data which is submitted by the scientific community. Thus GEO data base is the combination of "geo" that is spatial data with "data base" that is data repository in order to create a central data repository for spatial data storage and management. In order to provide help to users query and download experiments and in order to collect the gene expression profile stored in GEO, a collection of web based interfaces and applications are available. Raw microarray data found in GEO database have images that can be converted into gene expression matrices where rows represent genes and column represent various samples. In order to extract any biological process information analysis of matrices can be done. Platform generally describes list of features on array.

The mRNA and miRNA expression data of the cancer fibrosarcoma and corresponding normal control fat sample was taken for our analysis.

PLATFORMS taken:

Expression Data	GEO accession	Title
mRNA	GPL96	[HG-U133A] Affymetrix Human Genome U133A Array
miRNA	GPL8179	Illumina Human v2 MicroRNA expression beadchip

Samples taken:

Expression Data	GEO accession	Title
mRNA	GSE21122	Whole-transcript expression data for soft-tissue sarcoma tumors and control normal fat specimens
miRNA	GSE36982	MicroRNA profiling of primary high-grade soft tissue sarcomas and control normal fat

2) Microarray analysis of gene Expression Data:

A) *Retrieval of mRNA and miRNA gene expression data*:

- All samples of fibrosarcoma and normal control fat (i.e. all GSMs) present in the GSE were taken for analysis
- The details of number of samples taken for mRNA and miRNA expression analysis are given in Table 3.
- The data were taken in multiples to reduce error rate.
- The raw files were downloaded in .CEL format.
- Then the files were unzipped, extracted and renamed as control and test for mRNA and miRNA accordingly.

Table 3. Number of samples taken for mRNA and miRNA expression analysis

Expression data	Sarcoma	No. of samples
mRNA	MFH:Myxofibrosarcoma	31
mRNA	Normal fat	9
miRNA	Myxofibrosarcoma	8
miRNA	Normal fat	2

B) *Analysis of Gene Expression Data*:

Agilent's GeneSpring GX 12.6 software is used for gene expression analysis. This software is a powerful microarray expression data analysis tool for fast visualization and analysis of different microarrays (gene, miRNA, exon, genome copy number, etc). Class of transcripts showing expression pattern which are correlated with experiment variables are identified by using this software. Thus mainly for fulfilling the needs of biologists this tool is designed.

Performing work in GeneSpring GX 12.6 is organized into projects. In a project a number of experiments are involved and again an experiment is composed of samples that are data sources. Samples are grouped as test and control and interpretations are done based on the defined parameters of sample grouping and finally analysis involves a number of statistical steps and corresponding results.

All information on array design along with biological information is found in Genespring GX. So for each new array type which is to be analyzed, a new technology must be installed initially. Technologies created include affymetrix for standard arrays, Agilent and Illumina.

In GeneSpring following steps are used:

I. For gene expression analysis :

In order to minimize the systematic non-biological differences to reveal true biological differences, normalization of data was done, after which profile plot of normalized intensity map values are obtained. To standardize each chip for cross comparison, data is normalized to 75^{th} percentile of signals intensity. The main purpose is to eliminate redundancy and make sure that the data makes sense with minimum number of entities.

A new experiment was created for analysis of gene expression. The experiment type should be specified as Affymetrix. Workflow selected was Guided Workflow.

Using choose sample option the unzipped samples (both sarcoma and normal fat) were uploaded to the experiment in GeneSpring from the saved location in the system. Experimental grouping was done to define samples as test and control and assigning a parameter name (e.g. Average).

By using Filter Probsets by Errors, quality control of samples was done. This was done on the raw signal values of all the entities. For filtering , cut off was set at 20 percentile of all intensity values and a profile plot of filtered entities was generated. Using the normalized signal values and grouped samples by active interpretation box whisker plot is generated.

Significance analysis depending upon experiment grouping, was done by performing T-Test unpaired analysis as 2 groups are there that is control and test along with replicates.

For computing p-values Benjamini-Hochberg FDR algorithm multiple testing correction was used. The p-value cut off taken was ≤ 0.05. This reduces the number of false positives or false discovery rate. This multiple testing correction is least stringent. Fewer chances of false negative genes are there. A p-value of ≤ 0.05 is taken as significant.

For identifying differentially genes among the cancerous and non-cancerous samples which are expressed above a definite threshold, fold change analysis is done. It gives the absolute ratio of normalized intensities between the average intensities of grouped sample. Fold change cut off is taken ≥ 2.0.

Further 2D hierarchical clustering of the genes expressed ≥ 2.0 fold was carried out taking average linkage to classify the cancerous and normal control samples and a heat map was generated using CLUSTER 3.0 and JAVA tree view. Hierarchical clustering method arranges gene in a tree structure based on their similarity. If the items are similar to each other then they are connected by short branches and if it is dissimilar then it is connected by long braches.

Genes expressed greater than 2 fold were exported from GeneSpring along with normalized signal values, gene symbols, entrez gene IDs etc. The entity list was exported as .txt file and later opened with excel for further analysis.

II. For miRNA expression analysis :

miRNA expression analysis was carried out in the same project of GeneSpring, but in a new experiment. miRNA specific platform i.e Illumina was chosen and further steps of analysis were same as that of mRNA expression analysis

The miRNAs differentially expressed greater than 2 fold between the normal and cancerous samples were exported for further analysis.

C) Analysis of Gene and miRNA list:

With the aim of finding a probable oncogene the gene ontology analysis was carried out for the genes up-regulated using web based gene set analysis tool kit. Genes with functions and probable role in metastasis was focused on.

Similarly with the aim of finding a probable tumor suppressor which might be targeting the oncogene of our interest the list of down-regulated miRNAs involved in fibrosarcoma was analyzed for its fold change value and function from literature study.

3) Target interaction map through Magia^2 Software:

The integrated analysis of in-silico target prediction of miRNA and gene expression data for reconstruction of post transcriptional regulatory networks is performed by using software called Magia^2. Due to different levels of regulation and highly interconnected networks of regulatory elements and their interaction, gene expression profile is resulted. Magia is a web based tool which is designed in order to cope with low sensitivity of target prediction algorithms by exploring the interaction of target prediction with miRNA and gene expression profiles. In order to improve detection of miRNA-mRNA for in-silico target prediction magia generates a highly interconnected network of miRNA, mRNA, transcription factors.

The gene expression and miRNA expression data were uploaded and submitted using Pearson correlation method analysis which aims to display the target interaction map for matched miRNAs and gene expression data.

From the interaction map one gene and one miRNA pair was selected based upon their regulation and association with cancer for further validation by qRT-PCR. This pair has not been validated experimentally in any cancer system before.

4) RNAhybrid study:

RNAhybrid was run to study interaction among the chosen gene and miRNA pair. It is a tool for finding the minimum free energy of hybridisation of a long and a short RNA.

5) Experimental validation :

Human fibrosarcoma cell line HT-1080 was obtained from National Centre for Cell Science, Pune, India. The media used for culturing the cell is DMEM (Dulbecco's Modified Eagle's Medium) with FBS (Fetal Bovine Serum) from HIMEDIA and 1% antibiotic solution. The culture flask containing the cell line is kept in CO_2 maintained at 5%. By utilizing medium, the color of medium changes from red to orange and then to pale yellow because of change in pH of medium.

CELL CULTURE:

I. Reviving of stored cells

For reviving cells they are first thawed by drastically reducing the stored cells temperature from -80^{o}C to 37^{o}C in a water bath to avoid formation of crystals. 5ml of DMEM was taken in a T25 flask and the thawed cell specimen is pipetted into the flask. The flask was swirled gently in order to mix the cells with medium. Sometime removal of DMSO is necessary especially for specimen cells, primary cells and sensitive cells types. For such cell types, pipette the thawed cell specimen into a sterile centrifuge tube containing medium. Centrifuge at 1500rpm for 5min, aspirate the supernatant, then re-suspend the cells in fresh medium. Cells were incubated overnight under their usual growth conditions. 24hrs later the cells were checked for their adherence to the flask and the growth medium was replaced. The cells are grown in a flask until they are confluent enough or their medium has been utilized and they are ready for sub-culture.

II. Trypsinizing cells:

Trypsinization is a technique that uses the proteolytic enzyme trypsin in order to detach adherent cells from the surface of a cell cultured vessel. This procedure is preferred whenever the cells need to be harvested and sub-cultured. For trypsinization the medium was aspirated and discarded. Cells were washed with 1X PBS. 1ml 1X trypsin EDTA solution was added. The flask was placed in a co2 incubator at 37^{o}c for 1 to 2 minute or until all cells were detached. Flask was removed from incubator and firmly taped with palm of hand to assist detachment. Once all the cells dislodged, cells were re-suspended in a growth medium containing serum. Use medium containing the same percentage of serum as used for growing the cells. The serum inactivates

trypsin activity. Then cells were pippetted gently up and down and transformed to an RNAase free glass or polypropylene centrifuge tube and then centrifugation done at 1500rpm for 5 minute. Then the supernatant was aspirated completely and the cells again re-suspended in 1X PBS and were counted. The appropriate no. of cells were taken for isolation of RNA by again pelleting the cells and removing PBS.

RNA isolation:

- **mRNA isolation** :

For mRNA isolation QIAGEN kit is used.

1) First at a maximum of 1×10^7 were harvested then appropriate volume of buffer RLT was added.

2) To the lysate, 1 volume of 70% ethanol was added and mixed well by pipetting. Immediately proceed to step 3.

3) 700µl of sample including any precipitation was transferred to an RNeasy mini spin column placed in 2ml supplied collection tube.Then the lid was closed and centrifuged for 15 sec at ≥ 8000xg.Then flow through was discarded.

4) To RNeasy spin column 700µl of RW1 was added. Then the lid was closed and centrifuged for 15 sec at 8000xg.Then flow through was discarded.

5) To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifuged for 15 sec at ≥ 8000xg.Then flow through was discarded

6) To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifuged for 2 minute at ≥ 8000xg.

7) In a new 1.5ml collection tube the RNeasy spin column was placed. 30-40µl RNase free water was added directly to spin column membrane. Then the lid was closed and centrifuged for 1 minute at ≥ 8000xg in order to elute RNA.

8) If the expected RNA yield is more than 30µg, then the step-7 was repeated using another 30-50µl of RNase free water or using the elute from step-7.The collection tube were reused from step-7.

9) By using Eppendorf NanDrop, the purity and yield of RNA was measured. It is a cuvette free spectrophotometer eliminating the need for other sample containment device and also allows for clean up in seconds. 1µl of sample can be measured with this instrument with high accuracy and reproducibility. 1µl of sample was pipetted onto the end of a fiber optic cable. A second fiber optic cable is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. A pulsed xenon flash lamp provides the light source and spectrometer utilizing a linear CCD array is used to analyze the light after passing through sample. The instrument is controlled by based software and the data is logged in an archive file on PC.

- **MicroRNA isolation** :

1) Cells were collected after trypsinization. And then wash with PBS and cells pellets were collected, then it was kept in ice for some minute.

2) PBS was removed, 600 µl lysis or binding solution was added.

(If the cells are 100s in number -300 µl)

(if cells are 1000s in number -600 µl)

3) Now the solution was vortex vigorously to make homogenous lysate.

4) $1/10^{th}$ (for 600/10=60 µl) of microRNA homogenate additions was added to cell, then mixed well by vortexing or inverting several times , then kept on ice for 10 minute.

5) 1 volume of Acid phenol chloroform that is equal to lysate volume is added before addition of miRNA homogenate, then vortexed for 30 to 60 sec in order to mix then it was centrifuged for 5 min at 10,000 g at room temperature to separate aqueous and organic phases. (After centrifugation the interphase should be compact if it is not then centrifugation is repeated)

6) Aqueous phase is carefully removed without disturbing the lower phase and transferred into a new tube.

7) 1.25 volume of room temperature 100% ethanol was added to aqueous phase.

8) A filter cartridge was placed into one of the collection tubes, then it was mixed by pipetting then centrifuged for 15 sec at 10000 rpm, then flow through was discarded until all the lysate is through.

9) 700µl of miRNA wash solution I was added to filter cartridge and then centrifuged for 5 to 10 sec and then flow through was discarded.

10) 500 µl of wash solution 2/3 centrifuged it for 5 to 10 sec. Again repeat it with 500 µl of wash solution 2/3.

11) After discarding the flow through from last wash, the filter cartridge was replaced same in collection tube and assembly was undergo spinning for 1 min to remove residual fluid from filter.

11) Filter cartridge was transferred into a fresh collection tube .100µL of pre heated (95^{o}C) elution buffer or nuclease free water was applied ,cap was closed and it undergo spinning for 20 to 30 sec at maximum speed to remove microRNA.

12) The elute was collected and stored at -20^{o}c or below.

cDNA Synthesis:

mRNA cDNA synthesis: cDNA Synthesis was carried out using Super Script First Standard Synthesis System for RT-PCR by invitrogen using oligo dT primer.

1) Each of the components were mixed and centrifuged briefly before use.

2) For each reaction the following in a sterile 0.2 or 0.5 tube was combined.

Components	Amount
RNA	4 µl
10mM dNTP mix	1 µl
Primer(0.5µg/µl oligo $(dT)_{12-18}$OR 2 µM gene specific primer)	1 µl
DEPC treated water	4µl

3) The RNA/Primer mixture was incubated at 65°c for 5 minute and then placed on ice for at least 1 minute.

4) Following 2X reaction was prepared in a separation tube by adding each component in the indicated order.

Component	1RXn	10RXn
10X RT buffer	2 µl	20 µl
25Mm $Mgcl_2$	4 µl	40 µl
0.1MDTT	2 µl	20 µl
RNase out™(400/µL)	1 µl	10µl

5) To each RNA/Primer from step3, 9µl of 2X reaction mixture was added, mixed gently and collected by a brief centrifuge.

6) It was incubated at 42°c for 2 minute.

7)1µl of superscript TM II RT was added to each tube.

8) It was incubated for 50 minute at 42°c.

9) The reaction was terminated at 70^{o}c for 15 minute and then chilled on ice.

10) By brief centrifugation the reaction was collected. To each tube 1 µl of RNase H was added and incubated at 37^{o}c for 20 minute and then the reaction was used for PCR immediately.

MicroRNA cDNA synthesis: miRNA cDNA synthesis was carried out using NCode™ miRNA First-Strand cDNA Synthesis Kit.

1) 100 to 1µg of optimal RNA was taken.

2) DNase I may be used to eliminate genomic cDNA contain.

3) Undiluted cDNA are to be used for qRT-PCR.

4) The following reaction volume may be scaled as needed upto 100µl .For single reaction , combine the following components in a tube on ice. For a multiple reactions a master mix is prepared without RNA.

Components	Amount
5X reaction mix	4 µl
10X superscript	2 µl
Total RNA	2.5 µl
DEPC water	11.5µl

5) The tube was capped then gently vortexed to mix and centrifuge then briefly.

6) The tube was then incubated at 37^{o}c for 60 minute, terminated at 95^{o}c for 5 minute and then hold the reaction at 4^{o}c until use.

SEMI-QUANTITATIVE RT-PCR ANALYSIS:

To assess the quality of the cDNA formed and to find the annealing temperature of the primer a semi-quantitative gradient PCR was carried out. The cycling conditions were 95 ^{o}C for 5 min for initial denaturation, 95^{o}C for 30secs for denaturation, 57^{o}C-60.6 ^{o}C for 60 sec as the range for annealing temperature, 72 ^{o}C for 1 min for final extension temperature, repeated 37 times.

QUANTATIVE RT-PCR ANALYSIS:

In qRT-PCR florescence signal is continuously collected from one or more polymerase chain reactions over a range of cycles. Thus florescence signals from each reaction are converted into a numerical value for each sample. In order to bind DNA florescent maker is used. Thus during the reaction as the number of copies increase, so the florescent intensity increases. SYBR green is a intercalating florescent dye, and it is the simplest and cheapest way in order to monitor a PCR in real time. The SYBR green dye florescence only on binding to double stranded DNA. Using dye has one disadvantage, which is lack of specificity.

From primer bank Database (Harvard) the gene specific primer sequence were obtained and the primers were synthesized by Sigma. All the primers were desalted.

Procedure of mRNA qRT-PCR:

In order to perform PCR by using RNA as a starting template, it must first have to transcribed into cDNA in a reverse transcription reaction, in which cDNA is act as template for RT-PCR with gene specific primres (Table 4.).

Table 4. Primer name and sequence with length and its amplicon size

Gene	**Sequence**	**Amplicon size**
ACTB	F- F-CATGTACGTTGCTATCCAGGC	250
	R- R-CTCCTTAATGTCACGCACGAT	
p15PAF	F- F-ATGGTGCGGACTAAAGCAGAC	123

	R- R-CCTCGATGAAACTGATGTCGAAT	
miRNA-429	F-CGGCGTAATACTGTCTGGTAAAACCGT	22

In Eppendorf Masterplex Real Time PCR, RT-PCR was carried out. Primer concentration was normalized and gene specific forward and reverse primer pair was mixed. Each primer that is forward and reverse primer concentration in mixture was 3.5µl.

a) Now the experiment was ready and the following PCR program was made on. A copy of the set up file was saved and all other PCR cycles were deleted. Threshold frequency was taken was 33%. The temperature cycle were taken as follows:

Stage	Temperature(^{o}c)	Time	Cycle
Stage 1	95	20 sec	1
Stage2	95 55 68	15 sec 15 sec 20 sec	40
Stage3	95 60 95	15sec 15sec 15sec	1

b) cDNA was diluted to 1:20 ratio concentration and then primer was added.

c) 10µl of a real time PCR reaction volume was made.

d) In each optional tube as the following mixture was made follows.

SYBR Green Mix(2x)	35µl

cDNA stock(cDNA:dH_2O[1:20])	40 µl
Primer pair mix(3.5µl each primer)	7 µl

e) With the help of in-built software , the RT-PCR result was analyzed.

f) The tubes were removed from the machine, after PCR is finished.

Procedure of microRNA-qRT-PCR:

a) Undiluted cDNA was used for quantification.

b) 10µl of a real time PCR reaction volume was made.

In each optional tube as the following mixture was made follows:

SYBR Green Mix(2x)	5 µl
cDNA stock	2µl
DEPC H_2O	2.6 µl
Forward primer	0.2 µl
Reverse Primer	0.2 µl

c) With the help of in-built software, the RT-PCR result was analyzed.

d) The tubes were removed from the machine, after PCR is finished.

RESULTS AND DISCUSSION

Microarray Analysis

- **mRNA Expression Analysis:** 1068 differentially expressed genes (DE) were obtained between Myxofibrosarcoma and the control normal fat samples. Of these 558 genes were up-regulated and 510 genes were down-regulated. Hierarchical clustering of the DE genes showed the relatedness among the samples as all control samples clustered together and all sarcoma samples clustered together (Fig 4).

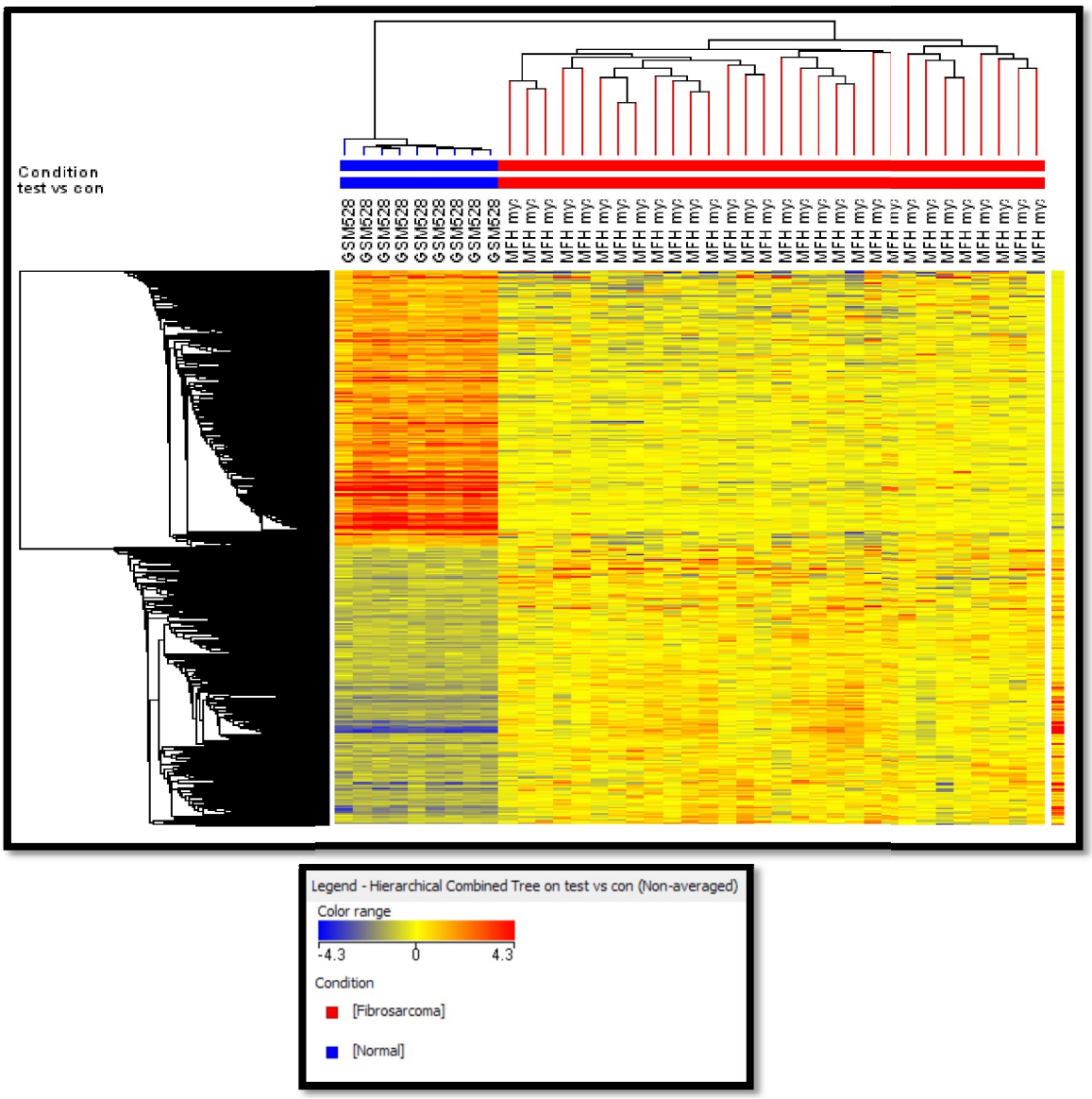

Fig 4: Hierarchical clustering of MFS and normal fat samples

- **microRNA Expression Analysis**: 77 differentially expressed miRNAs were obtained between Myxofibrosarcoma and the control normal fat samples. Of these 51 miRNAs were up-regulated and 27 miRNAs were down-regulated. Hierarchical clustering of the DE miRNAs showed the relatedness among the samples as all control samples clustered together and all sarcoma samples clustered together (Fig 5)

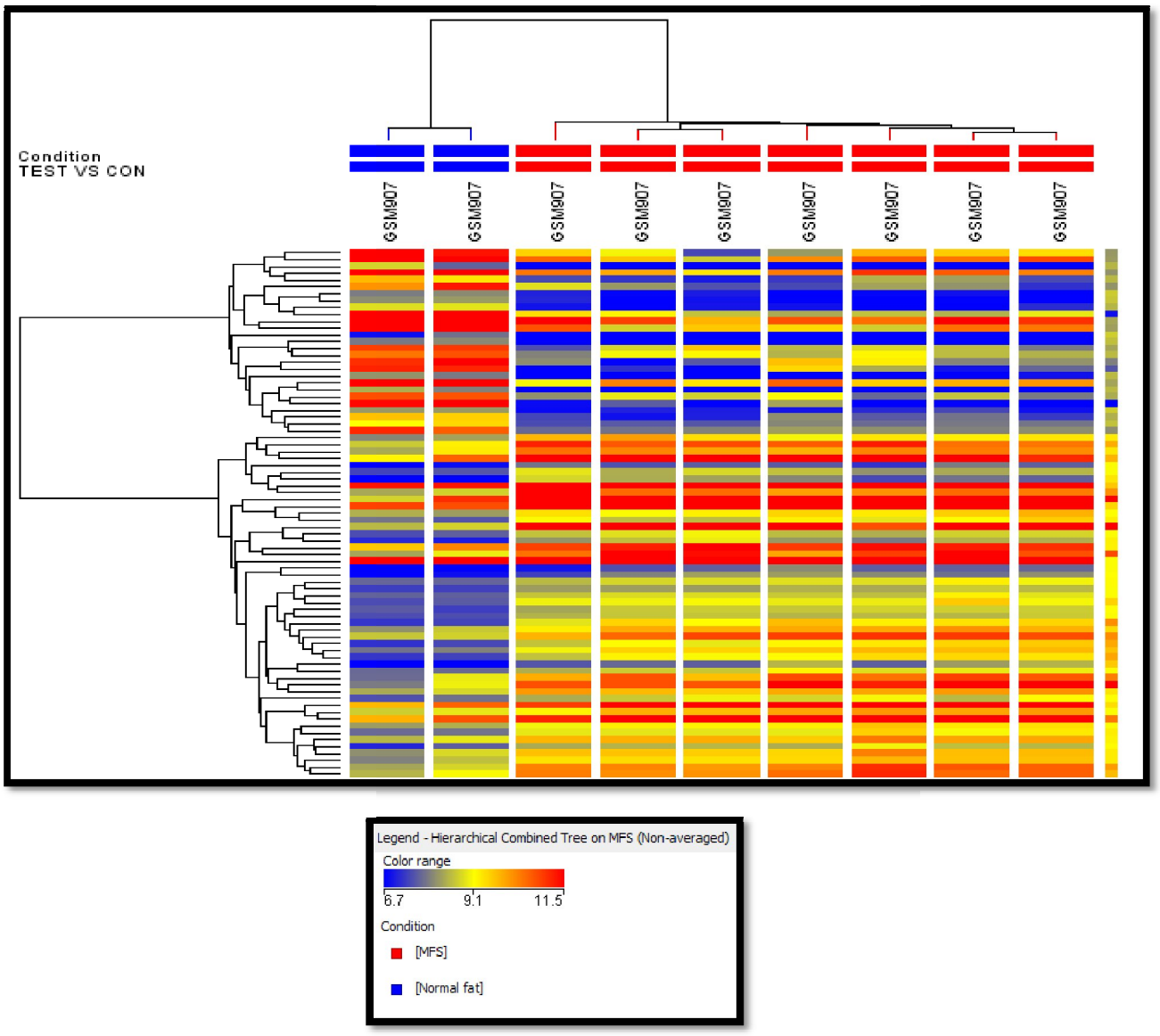

Fig 5: Hierarchical clustering of differentially expressed miRNAs

GO analysis: GO analysis was carried out using a web based gene set analysis tool kit to find the genes involved in different biological processes. As we wanted to find a gene involved in the

metastasis of sarcomas we mainly focused on the genes involved in the cell proliferation process which leads to metastasis (Fig 6).

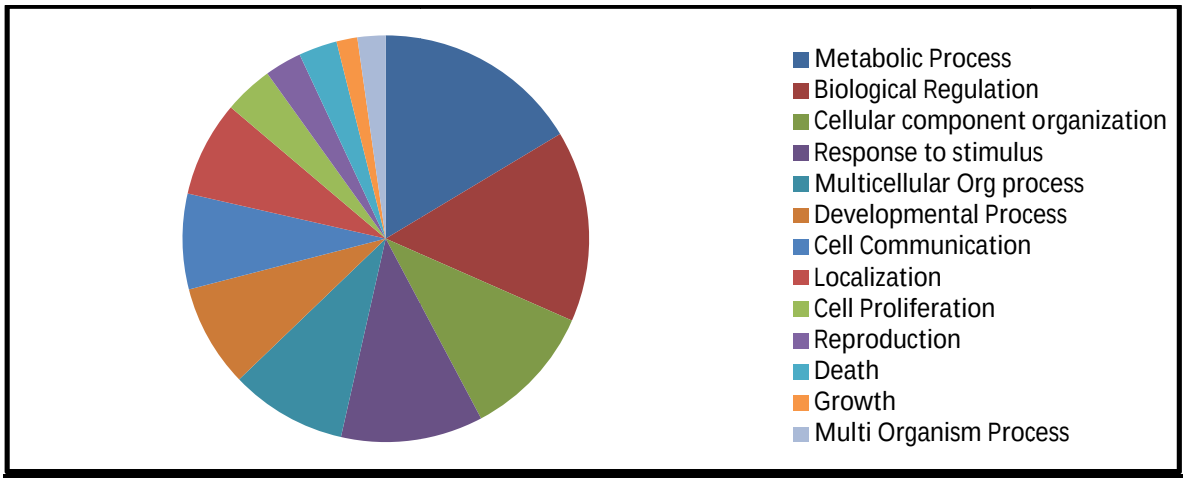

Fig 6: GO analysis of differentially expressed genes in fibrosarcoma

From the genes involved in cell proliferation, genes which were up-regulated were considered for further analysis for interaction with miRNAs which are down-regulated and have a probable tumor suppressive role.

<u>Target interaction map analysis:</u> Target interaction map resulted in 36 genes targeted by the miR-429 and 4 genes by the miR-375.

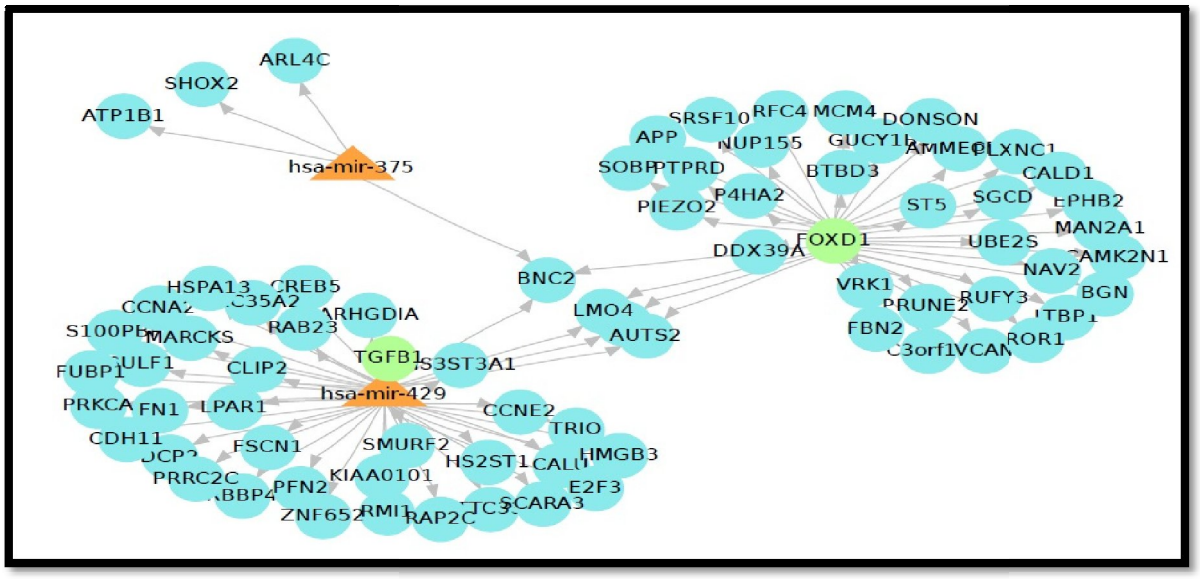

Fig 7: Interaction map of mRNA and miRNAs involved in fibrosarcoma

From the two miRNAs obtained from the target interaction map, miR-429 was selected based on its role in cell proliferation and migration of cancer cells as obtained from extensive literature study and high fold down-regulation obtained in all sarcomas according to microarray data. This showed that it might act as a tumor suppressor in the sarcomas. From the 36 genes it targeted we selected 10 genes based on their fold change and literature analysis for probable role as an oncogene.

RNAhybrid

RNAhybrid interaction was carried out using the miR-429 and all 10 genes and the p15PAF gene targeted by the hsa-miR-429 was selected. This gene and miRNA pair was selected based on the q-value of the pair which was found to be lowest from the Magia[2] interaction analysis. Other factors considered for choosing the pair was their fold change information from microarray data, extensive literature study on their probable role in other cancers and finally the lowest MFE (minimum free energy) value found among all the 10 pairs and a good 8 mer binding.

Interaction between p15PAF (3′UTR) and miR-429 using RNAhybrid

In this result we found that the **p15PAF** form hybrid with **has-miR-429** at position 22067 and eight nucleotide pairing (8 mer) was found in the RNA hybrid.

```
TARGET : gi|568815583:c64387687-64364994
length: 22694
MIRNA  : hsa-miR-429
length: 22

mfe: -15.2 kcal/mol
p-value: undefined

position  22067
target 5'    U      A  UUAAAAAAUU        A 3'
               UUUUGC CA          CAGUAUUA
               AAAAUG GU          GUCAUAAU
miRNA  3' UGCC         CU                 5'
```

Experimental Validation:

RNA Isolation

260/280 ratio: This indicates the absorbance of RNA and DNA at 260 nm and 280 nm respectively. It is used to access the purity of DNA and RNA. Approximately 1:8 ratio is generally expected and accepted as "pure" for DNA and approximately 2.0 ratio is as "pure" for RNA. In either case, if the ratio is significantly lower it may indicate the presence of phenol, protein or other contaminants that absorb strongly at or near 280nm.

260/230 ratio: It is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid is generally higher than the respective 260/280 values. Expected 260/230 values are normally lies in the range of 2.0-2.2. If the ratio is significantly lower than the expected ratio, then it indicatse that contaminants absorbing at 230 nm is present.

Here from 2 samples we got following result:

HT1080 mRNA conc. = 254.1µg/ml	H1080 miRNA conc = 400.1µg/ml
At (260/280) = 2.07	At (260/280) = 2.00
At (260/230) = 1.79	At (260/230) = 2.01

Semi-quantitative PCR

A semi quantitative gradient PCR was carried out to assess the annealing temperature of the gene p15PAF. Also we could know the quality f the cDNA prior to setting up qRT-PCR and get an idea about the quantitative amount of the gene with respect to beta actin and thus called semi-quantitative **(**Fig 8).

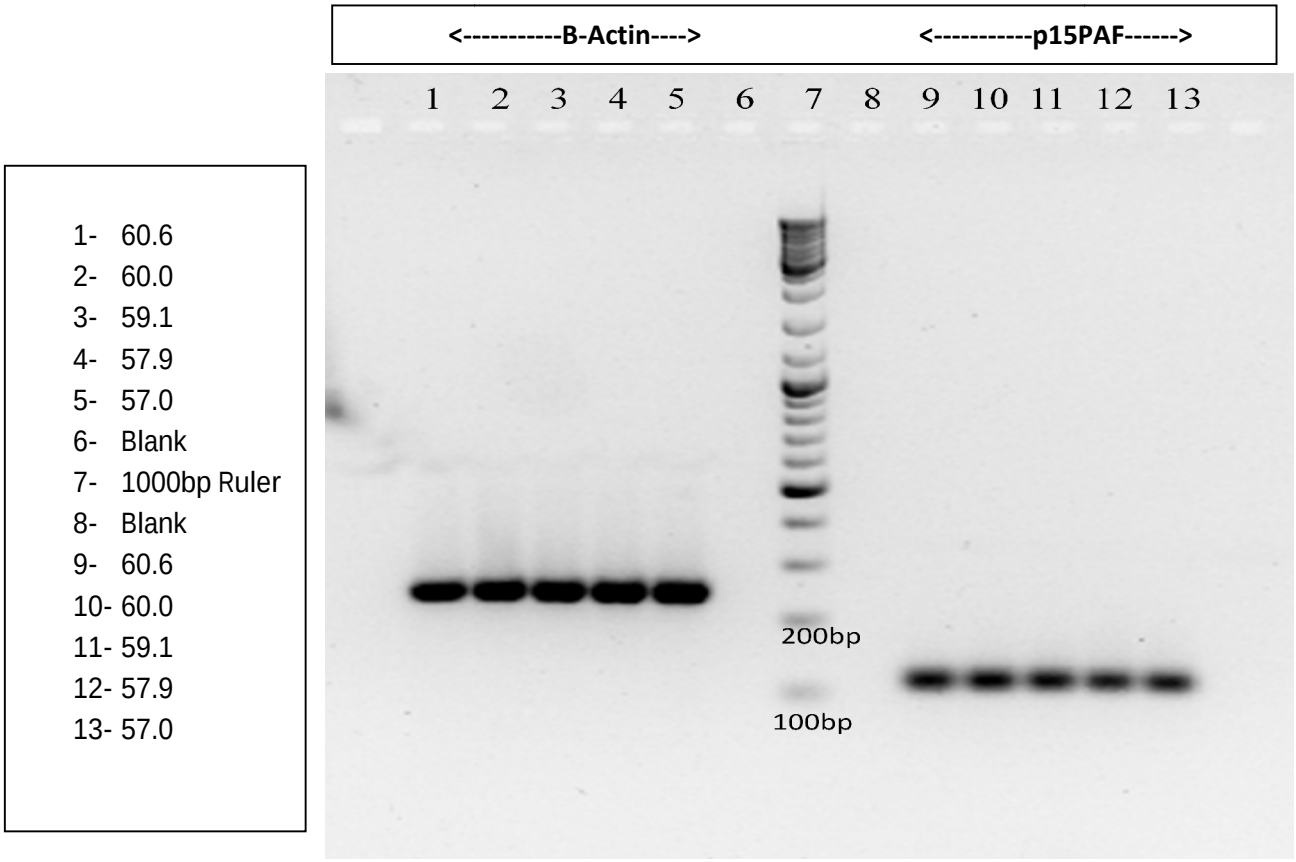

Fig 8: Result of Semi-quantitative PCR

qRT-PCR:

The qRT-PCR program used 59ºC as annealing temperature as the amplification for ACTB and p15PAF was best at that temperature (Fig 9).

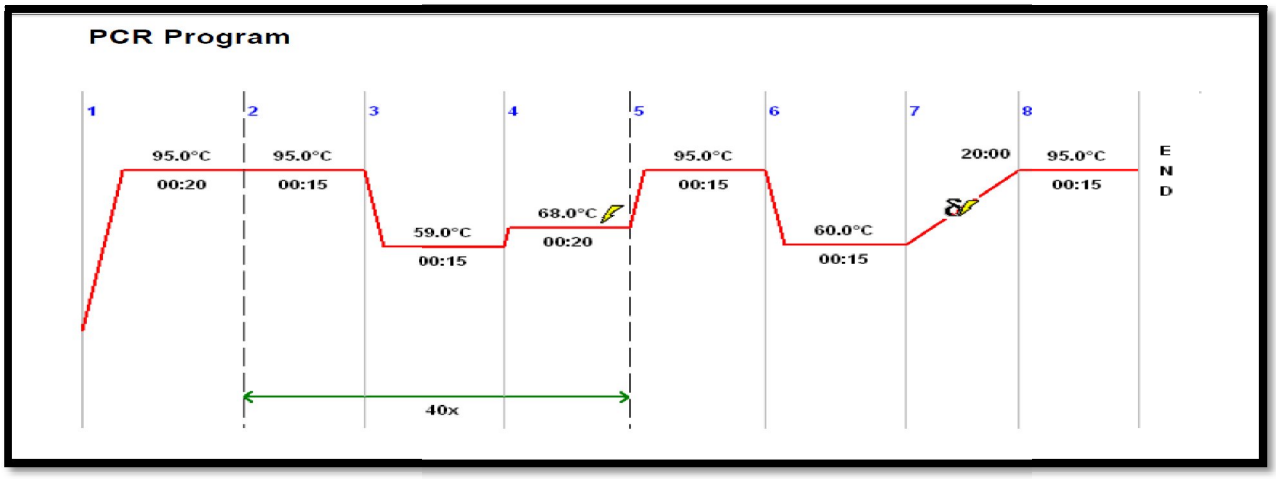

Fig 9: Cycle temperature and time for qRT-PCR

- qRT-PCR melting curve analysis showed a single amplification product was formed for the p15PAF and ACTB as only a single peak was obtained for the respective genes. There was no non specific amplification (Fig 10).

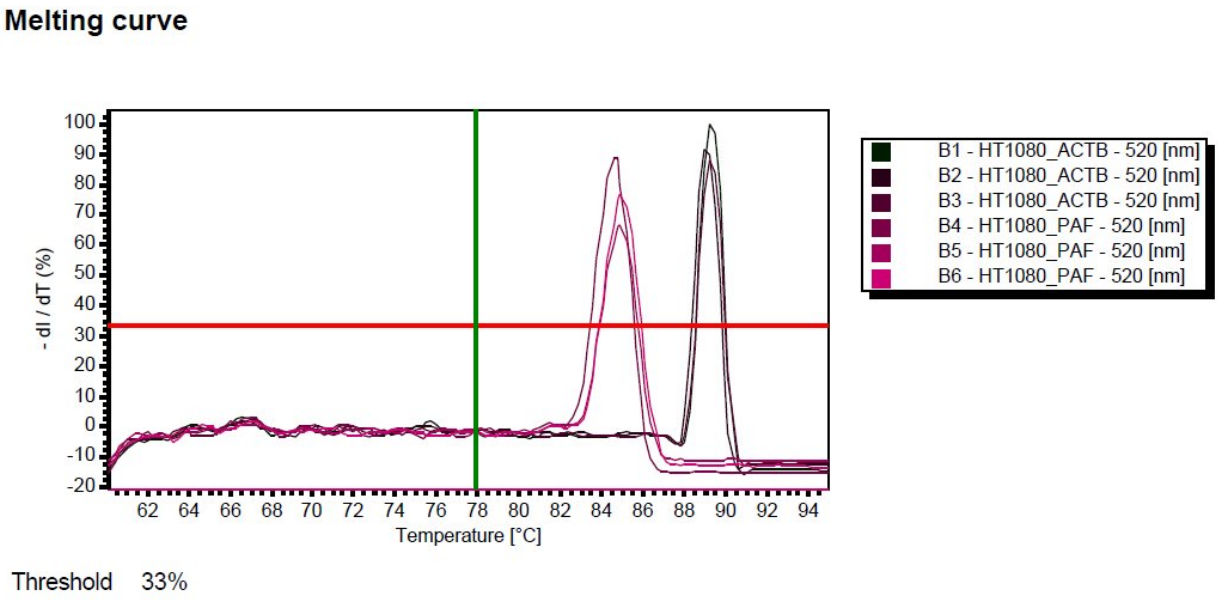

Fig 10: Melting temperature curve of p15PAF with respect to ACTB

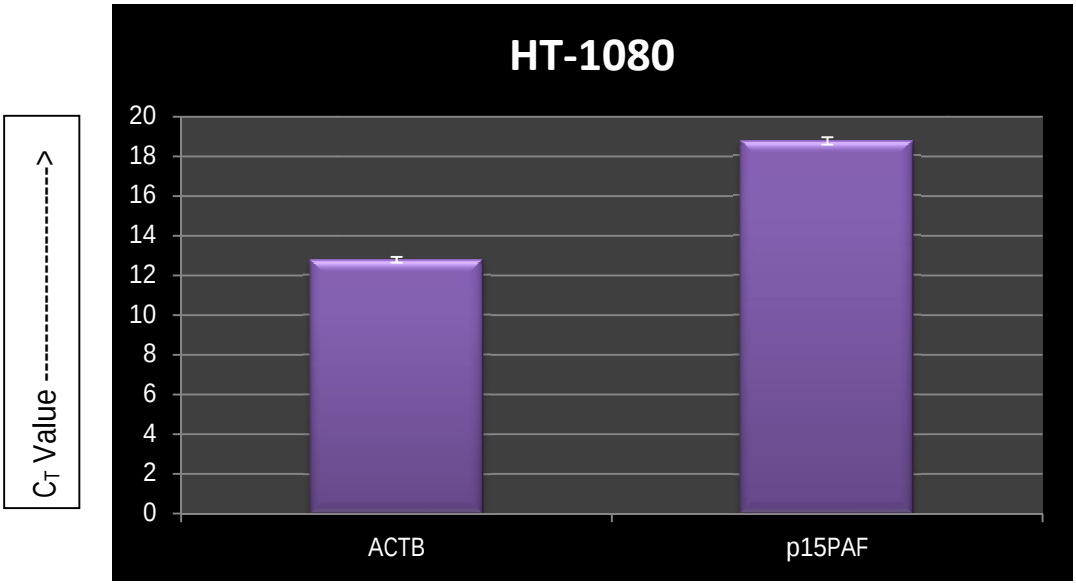

Fig 11: Result of qRT-PCR analysis of p15PAF gene and miR-429

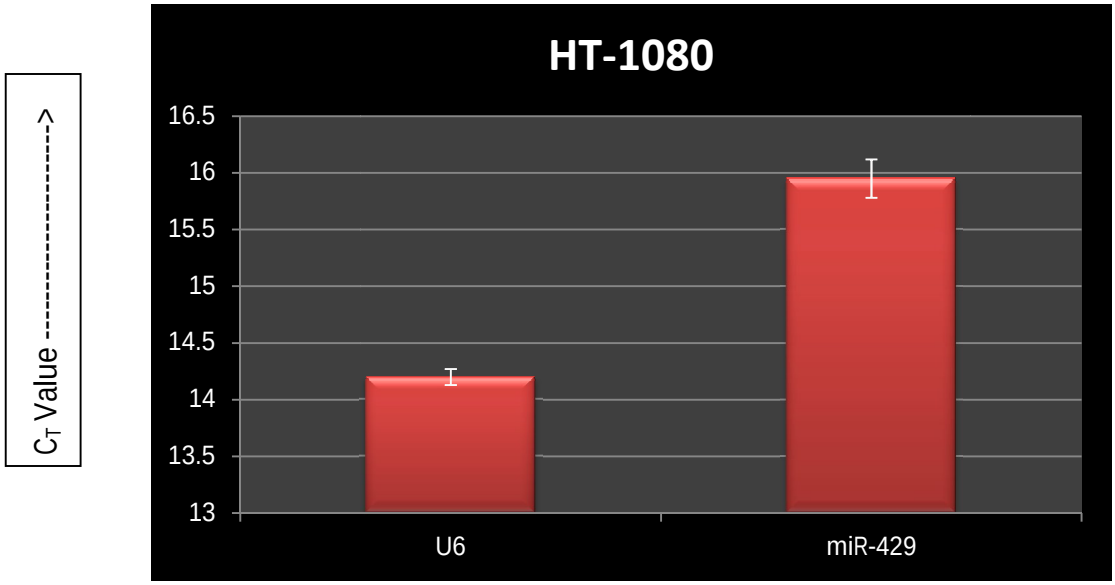

Fig 12: qRT-PCR analysis of p15PAF gene and miR-429

From qRT-PCR analysis of p15PAF gene, a fine melting curve of p15PAF is observed in comparison to the control β-actin (Fig 11.). The melting temperature of p15PAF is 84.8ºC and ACTB is 89.2 ºC. As the samples of gene were taken in triplets, 3 peaks of p15PAF positioned at one place were observed. The quantification of p15PAF with respect β-actin shows lower expression of p15PAF. Similarly the relative quantification of miR-429 with respect to U6 snRNA shows a lower expression of miRNA-429 (Fig 12).

CONCLUSIONS

Our study aimed at recognition of novel mRNA-miRNA target pairs which is considered to play important role in fibrosarcoma through an mRNA-miRNA interaction map analysis of microarray data along with experimental validation of selected set of mRNAs. From mRNA microarray expression analysis, we found 1068 differentially expressed genes between Myxofibrosarcoma and the control normal fat samples. Again from miRNA expression analysis, 77 differentially expressed miRNAs were obtained between Myxofibrosarcoma and the control normal fat samples. Of these, 51 miRNAs were up-regulated and 27 miRNAs were down-regulated. From target interaction map we have chosen one pair, hsa-miR-429-p15PAF which was seen to be novel target pair. The quantification of p15PAF with respect β-actin shows lower expression of p15PAF. Similarly the relative quantification of miR-429 with respect to U6 snRNA shows a lower expression of miRNA-429. As we didn't have control cell line we couldn't go for relative gene expression analysis of the gene and miRNA. Further analysis of the expression of the gene and miRNA has to be carried out to establish their role as an oncogene and a tumor suppressor miRNA respectively. Further analysis of their role in metastasis has to be carried by their over-expression/ inhibition and carrying out using different cell proliferation assays like MTT assay and migration assays like wound healing and scratch assay. These assays will establish their role as an oncogene and tumor suppressor miRNAs. Further analysis can prove whether hsa-miR-429 and p15PAF can be considered to be novel target pair which will be playing a role in the metastasis of fibrosarcoma.

FUTURE PROSPECTIVES

The effectiveness of miRNA based fibrosarcoma therapy can be a land mark in fibrosarcoma studies. Recognition of genome wide targets of miRNAs is a promising approach which can be experimentally validated to have a role in fibrosarcoma. Henceforth, with a better prospective about the gene networks and their cellular pathways regulated by miRNAs, the elucidation of fibrosarcoma pathogenesis and therapeutics can be facilitated. Furthermore, experimental validation of hsa-miR-429- p15PAF through Luciferase Reporter Assay or any other molecular techniques will strengthen the foundation of miRNA – mediated regulation in fibrosarcoma. Subsequent analysis of this novel miRNA-target pair will enhance our understanding to manipulate pathways for treatment of fibrosarcoma through microRNA mediated therapeutics.

REFERENCES

1. D'Angelo SP, Tap WD, Schwartz GK, Carvajal RD. Sarcoma Immunotherapy: Past Approaches and Future Directions. Sarcoma. 2014; 2014:391967. Epub 2014 Mar 20.

2. Zambo I, Veselý K. [WHO classification of tumours of soft tissue and bone 2013: the main changes compared to the 3rd edition]. Cesk Patol. 2014 Apr;50(2):64-70.

3. Schultze J, Schwick B, Galalae R, Kimmig B. [Late results of radiotherapy of soft tissue sarcoma]. Rontgenpraxis. 1998;51(11):413-9.

4. Ainsworth KE, Chavhan GB, Gupta AA, Hopyan S, Taylor G. Congenital infantile fibrosarcoma: review of imaging features. Pediatr Radiol. 2014 Apr 6.

5. Than M, Han M. Functional analysis of the miRNA-mRNA interaction network in C.elegans. Worm. 2013 Oct 1;2(4):e26894.

6. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007 Feb 1;302(1):1-12. Epub 2006 Aug 16.

7. Appelbaum FR. The acute leukemias. In: Goldman L, Schafer AI, eds. *Goldman's Cecil Medicine*. 24th ed. Philadelphia, PA: Elsevier Saunders; 2011:chap 189.

8. Kyle RA, Rajkumar SV. Treatment of multiple myeloma: a comprehensive review.Clin Lymphoma Myeloma. 2009 Aug;9(4):278-88.

9. Loh ML, Ahn P, Perez-Atayde AR, Gebhardt MC, Shamberger RC, Grier HE. Treatment of infantile fibrosarcoma with chemotherapy and surgery: results from the Dana-Farber Cancer Institute and Children's Hospital, Boston. J Pediatr Hematol Oncol. 2002 Dec;24(9):722-6.

10. Leber MF, Efferth T. Molecular principles of cancer invasion and metastasis (review). Int J Oncol. 2009 Apr;34(4):881-95.

11. Fokas E, Engenhart-Cabillic R, Daniilidis K, Rose F, An HX. Metastasis: them seed and soil theory gains identity. Cancer Metastasis Rev. 2007 Dec;26(3-4):705-15. Review.

12. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009 Apr;9(4):274-84.

13. Stoletov K, Kato H, Zardouzian E, Kelber J, Yang J, Shattil S, Klemke R. Visualizing extravasation dynamics of metastatic tumor cells. J Cell Sci. 2010 Jul 1;123(Pt 13):2332-41.

14. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev. 2009 Jun;28(1-2):15-33.

15. Schlesinger M, Bendas G. Vascular Cell Adhesion Molecule-1 (VCAM-1) – An increasing insight into its role in tumorigenicity and metastasis. Int J Cancer. 2014 Apr 26.

16. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res. 2008 May 15;68(10):3645-54

17. Albelda SM. Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab Invest. 1993 Jan;68(1):4-17.

18. Chambers AF, Matrisian LM. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst. 1997 Sep 3;89(17):1260-70. Review.

19. Cai Y, Yu X, Hu S, Yu J. A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics. 2009 Dec;7(4):147-54

20. Esquela-Kerscher A, Slack FJ. Oncomirs — microRNAs with a role in cancer. Nat Rev Cancer. 2006;6(4):259–269

21. Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer.Annu Rev Med. 2009;60:167-79

22. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell. 2003 Dec 26; 115(7):787-98.